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Assessing the health of blue mussels (*Mytilus edulis*) for site-selection of cultivation areas: potentials and constraints of applied parameters

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In a comprehensive approach intertidal, near- and offshore areas in the German Bight were evaluated to determine their suitability for the cultivation of blue mussels (*Mytilus edulis*). Over a ten month sampling period mussels were assessed using biodiagnostic marker -so far applied only in environmental monitoring- such as lysosomal membrane stability and the concentrations of lipofuscin, neutral lipids and glycogen in cells of the digestive gland. Together with investigations regarding the presence of macro- and micro parasites in mussel's tissues the overall health of animals was described. First results showed high condition indices, low parasite infestations and very high growth rates for submerged mussels. In contrast, intertidal mussels had significantly higher parasite loads and lower condition indices. A refers pattern was obtained regarding the concentration of e.g. neutral lipids in cells of the digestive gland. Due to the permanent feeding mode submerged mussels accumulated significantly more neutral lipids than intertidal mussels. However, these differences between sites were not displayed using screening tools sensitive for environmental impacts and individual stress such as the test on lysosomal membrane stability. Here, mussels from all sites showed comparably low, values throughout the sampling season. Differences were displayed only on inter seasonal level with e.g. low labilisation values in spring due to the reproduction of mussels. The results show that mussels may react differently to the same level of environmental stress according to their habitat conditions. Further, there is a need to evaluate impact and relevance of health related parameters to improve the significance of deployed parameters.

Keywords: *Mytilus edulis*, biodiagnostic techniques, health, offshore, cultivation

1. Introduction

Along the Germany Bight, most of the protected nearshore areas are comprised of natural reserves, recreational areas and shipping routes. An expansion of marine aquaculture in suitable coastal areas is limited, since many stakeholders with vital interests compete for the same space (Buck 2004). A shifting of the mussel production off the coast would minimise spatial conflicts, but requires different culture techniques, since the water is too deep for on-bottom cultivation in most offshore areas. Technical solutions are arising with the first positive results of off-bottom cultivation experiments showing that these techniques are a potential alternative to the traditional on-bottom cultivation (e.g. Kamerman et al. 2002, Walter & Liebezeit 2003) even under harsh hydrodynamic conditions (Langan & Horton 2003). The idea of relocating aquaculture systems offshore was provided momentum as a new stakeholder, the offshore wind farm industry entered the scene, offering a unique opportunity to co-use large marine areas (Buck 2002). A sharing of: the solid groundings of windmills to attach the culture systems (Buck 2002, Buck et al. 2006) have been proposed.

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However, compared to the traditional methods of mussel cultivation in Germany the offshore longline approach would be cost intensive. A realisation of this idea is perhaps desirable under the perspective of spatial efficiency and a sustainable expansion of extensive production of seafood, but it is not independent of economic restraints. Therefore Buck and Michler-Cieluch (2009) calculated a potential scenario of a virtual offshore mussel farm and determined the economics drivers. The two most important parameters making an offshore mussel farm profitable are the number of market sized mussels available per meter longline and the price achievable at the market. The first parameter presents a more technical aspect of how to design the artificial substrates that mussels settle on so that they will not be dislodged by the harsh hydrographical conditions. The second crucial point market price is highly dependent on the quality and optical appearance of the mussels. Only, healthy mussels living under the best conditions, free of growth hampering factors such as parasites and pollutants with a high meat yield and good shell optics, reach the highest price on the market.
In nearshore intertidal areas, mussels are particularly exposed to high concentrations of pollutants, pesticides, near surface agents and estuarine runoffs etc, which can pose a threat to the health of mussels. The scope of growth, i.e. the energy available for growth, is usually directly and positively correlated to a good overall health condition of the respective organism (Allen & Moore 2004). But organisms with high growth rates and an aesthetic appearance are no guarantee for a good overall health. In waters, eutrophicated and contaminated by urban sewage, mussels can show good growth performance, although they may carry high concentration of heavy metals and other pollutants.
In addition macro parasites living in blue mussels are numerous in intertidal and nearshore areas. Buck et al. (2005) have shown that offshore grown mussels were free of macro parasites. Infestation rates increased the closer the sites were to shore, where in particular intertidal mussels showed the highest numbers of parasites. The debate over the effects of macro parasites on the energy status and overall health of the host is still open, data needed to elucidate these issues are still lacking. The only micro parasite known to be associated with *M. edulis* along the European Atlantic coast is *Marteilia refrigens,* causing the Marteilliosis or Aber disease (Le Roux et al. 2001). From the North Sea, infested populations of mussels have been reported from the British Isles, whereas the eastern regions including the German Bight are regarded as micro parasite free. Marteilliosis in mussels is generally associated with a poor condition index, exhaustion of energy reserves (e.g. glycogen) and high mortalities (Grizel et al. 1974). Mass infections with *M. refrigens* can have a severe economic impact, e.g. oyster farmers in France lost about 440 million Euros in two years (1980 and 1983) due to Marteilliosis.

1.1. First trials to apply biodiagnostic tools in aquaculture

The presence of toxic compounds in the environment can be detected by chemical analysis of water and sediment samples. However this approach provides only minimal information on the effects of these toxic chemicals on biological systems. Analysing the chemistry of water or sediments does not provide information on concentrations of pollutants in organisms and their tissues. Chemical analysis of biota will provide only limited indications of health effects, since interactions or combination effects of pollutants are not covered with this approach. Therefore chemical analyses alone are inapplicable as cost-effective tools to detect e.g. "hot spots" of pollution (UNEP/ STAP 2003). As a result the health of so-called sentinel organisms were assessed to describe the quality of a certain environment the investigated animals lived in. Bivalves are the most commonly used sentinel organisms for the health assessment of the marine environment. The special properties of a sentinel species are that it is able to survive in a polluted habitat, and accumulates chemicals in its tissues. Due to their ability to accumulate and reflect a wide range of contaminants, mussels have been widely used in marine pollution monitoring (Goldberg 1975, Cajaraville et al. 1990, Livingstone et al. 1990, Smolders et al. 2003, Marigómez et al. 2006, ICES 2006). The blue mussel predominantly inhabits shores and estuarine environments. These habitats are very complex, varying in temperature, salinity, duration of exposure to air and food supply due to tides. To cope with these factors, the blue mussel has developed a series of behavioural, physiological and metabolic adaptations. Joergensen (1990) has described the mussel as an autonomous unit, incapable of regulation of its metabolism, meaning that physiological processes of the mussel respond directly to environmental changes.

The blue mussel is an active suspension feeder, filtering mainly phytoplankton from the water column. Due to this filtering mechanism, mussels ingest, besides phytoplankton, suspended particular material, bacteria, algae toxins and all kinds of pollutants and particles from their marine environment. As sessile organisms, they directly reflect the contaminant conditions of their habitat. As a result molluscs and especially blue mussels are the bioindicator of choice in several national and international biomonitoring programs e.g. MED POL (UNEP Mediterranean Biomonitoring Programme) or BEEP (EU Biological Effects of Environmental Pollution Programme).

Biomarkers can be deployed to assess the impacts of stress at the molecular and cellular levels, thus providing the earliest warning signals of toxic chemicals on tissues and organisms (Shugart et al. 1990; 1992). On the organism level, biomarkers can be used to indicate the potential survival capacity and the reproductive performance of the investigated animals. The latter is essential when relating the measured effects of individuals to possible changes for the population. According to their level of sensitivity biomarkers are classified into three main groups: biomarkers of exposure, biomarkers of genotoxicity and biomarkers of stress (Viarengo et al. 2007). In this study only biomarkers of stress were applied, which will be described in more details below. Stress sensitive biomarkers can be used to assess the health of an ecosystem as a whole in which the organisms live in (Cajaraville et al. 1998) or for the analysis of individual organisms that live in a specific environment or at specific contaminated sites. Well established examples of biomarkers of stress are the tests for lysosomal membrane stability, the lysosomal lipofuscin content and the neutral lipid accumulation in lysosomes.

Lysosomes are cell organelles containing various hydrolytic enzymes necessary for different metabolic processes surrounded by a semi permeable membrane (e.g. Moore 1976, Ferreira & Dolder 2003). They are responsible for the recycling of used-up cell organelles, macro molecules and metabolic waste products, and isolate harmful substances, once they have entered the cells. Lysosomes in molluscan digestive cells accumulate metals, organic contaminants as well as nanoparticles that cannot be degraded. These substances may provoke significant alterations in the lysosomes (Moore et al. 1980a, Moore et al. 1980b, Nott et al. 1985, Viarengo et al. 1985, Sarasquete et al. 1992, Cajaraville et al. 1995, Moore et al. 2004, Koehler et al. 2008). In general, contaminants from the environment cause a significant increase in size and number of lysosoms (Marigómez et al. 1989, Regoli et al. 1998, Koehler et al 2002). When that the storage capacities of lysosomes are overloaded and cells are stressed by high concentrations of harmful substances, the lysosomal membrane becomes instable and leaky. Pollutants and hydrolytic-lysosomal enzymes can re-enter the cytoplasm with serious risk of cell death (Koehler et al. 2002). When membrane stability and the over-all health status of mussels are low, more specific tests may elucidate the type and background of the infection or pollutant. Vice versa, if membranes of the lysosomes are stable there is strong evidence that the individual mussel grew under optimal water conditions (Widdows et al. 2002, Moore et al. 2004). Impairment of lysosomal functions and, hence, of food assimilation, can result in severe alterations in the nutritional status of cells and the whole organism, and could be indicative of disturbed health. For that reason, lysosomal changes and especially lysosomal membrane destabilisation are widely accepted as general stress biomarkers (Moore et al. 2004).

Glycogen is the molecule that functions as the secondary long-term energy storage in animal and fungi cells. Glycogen is found in the form of granules in the cytosol in many cell types, and plays an important role in the glucose cycle. Glycogen forms an energy reserve that can be quickly mobilized to meet a sudden need for glucose. In bivalves glycogen is the primary energy reserve (Patterson et al. 1999). Environmental stress and anthropogenic pollution is increasing stress for the mussel, thus leading to an increased metabolism and reduced glycogen storage. Since modifications of glycogen levels correlates with the level of chemical stress this parameter is used as a general biomarker for environmental monitoring (Ansaldo et al. 2006).

Lipofuscin, also known as age pigment, is widely regarded as end product of protein and lipid peroxidation due to oxidative stress (Au et al. 1999; Au 2004; Terman and Brunk 2004). Increased accumulation of lipofuscin in lysosoms of the digestive gland of mussels or in the liver of fish has been shown to be associated with contamination by anthropogenic pollutants e.g. heavy metals (Krishnakumar et al. 1994; Krishnakumar et al. 1997; Au et al. 1999; Au 2004). Accumulation of neutral lipids in cell vacuole is an indicator of lipidosis induced by toxic chemicals, especially due to exposure to xenobiotics such as polycyclic aromatic hydrocarbons (Lowe et al. 1981).

This study is the first trial to implement biodiagnostic tools for site selection and health monitoring in marine extensive aquaculture of mussels. As a priority a synchronic sampling throughout one annual cycle was conducted, where parameters of interest were investigated using the same individual (condition index, lysosomal membrane stability, lipofuscin, neutral lipids) or at least mussels from the same cohort (macro and micro parasites) for comparison and correlation. This information can be used to determine the suitability of site conditions and help calculate economic risks for potential mussel farmers.

2. Material and methods

Five locations along the coast of the German Bight were sampled to test and analyse mussels grown under different conditions (Fig. 1). Three areas were natural beds of mussels near Neuharlingersiel (NH, upper intertidal, Position 53° 42’ 10’’ N; 007° 43’ 50’’ E), Bordumer Sand (BS, upper intertidal, Position 53° 30’ 00’’ N; 008° 06’ 00’’ E) and from the Lister Strand from the Island of Sylt (SY, lower intertidal, Position 55° 01’ 32’’ N; 008° 26’ 43’’ E).

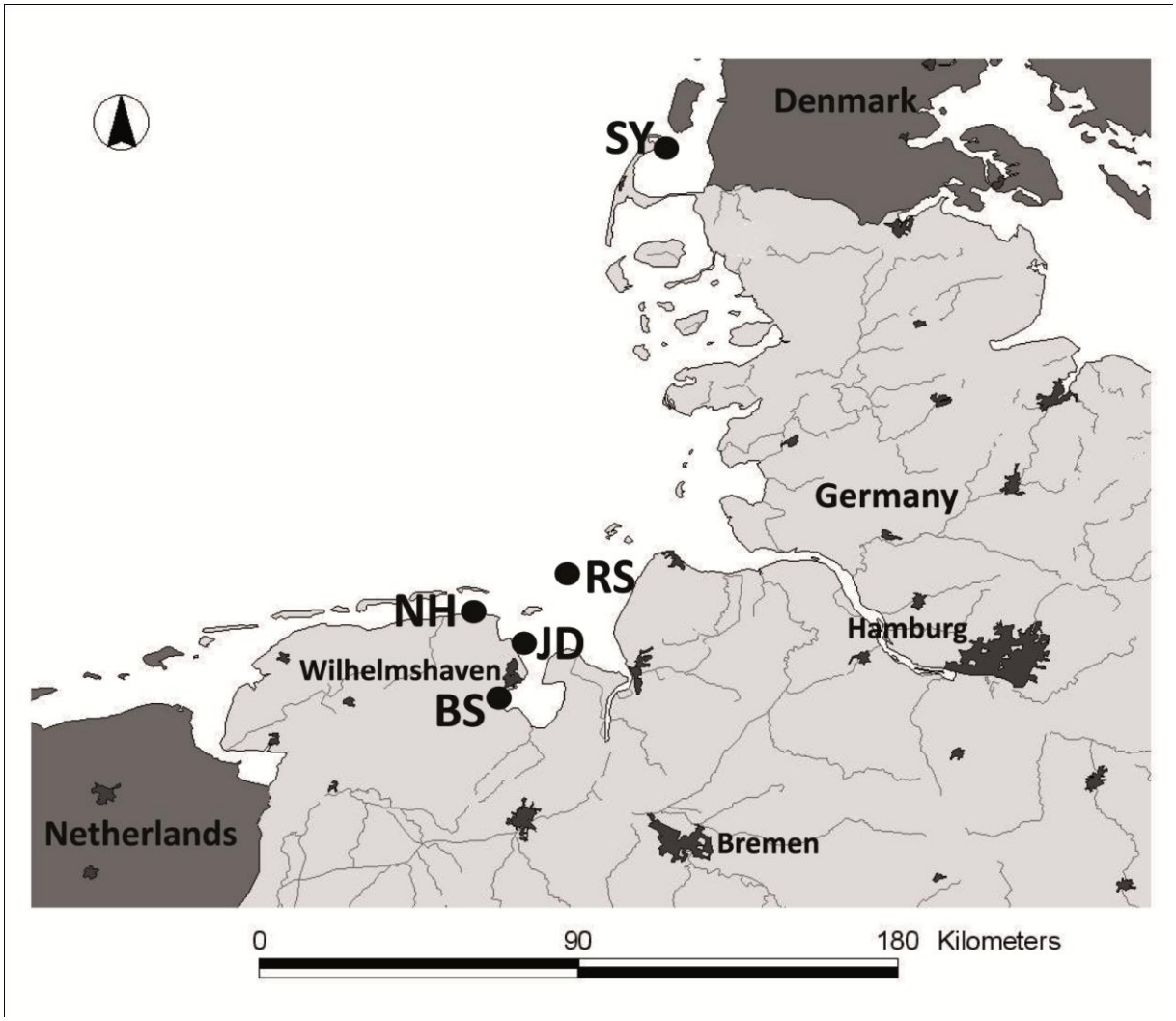

Fig.1: Map of the German Bight showing the sample sites. Three intertidal sampling locations at Neuharlingersiel (NH), Bordumer Sand (BS) and Lyster Strand at the island of Sylt (SY) and two suspended hanging cultures at the *Niedersachsenbrücke* (nearshore) near Wilhelmshaven in the Jade (JD) estuary and offshore at the entranceof the Weser estuary near the lighthouse *Roter Sand* (RS) were sampled in the year 2007.

Two locations were specially designed testing areas, where mussels were grown suspended on an artificial substrate: the nearshore location on the *Niedersachsenbrücke*, an approx. 1.300 m long cargo bridge, at the Jade bay (JD, Position 53° 35' 05'' N; 008° 09' 14'' E) near the city of Wilhelmshaven, and under offshore conditions an area called *Roter Sand* (RS, Position 53° 51' 00'' N; 008° 04' 20'' E) situated in the outer Weser estuary ca. 17 nautical miles northwest of the city of Bremerhaven. Throughout 2007 four consecutive sampling cycles in March, May, August, and November were conducted to test for site and seasonal influences on assessed parameters. Each sampling cycle was completed within 10 days and all parameters were analysed for each site and sample cycle. Intertidal areas (NH, BS and SY) were sampled at low water, whereas RS had to be sampled at slack water with a team of scuba divers operating from a research vessel. The JD site is accessible without any tidal constraints all year round. At each sampling site ca. 5 kg of mussels were collected for all investigations.

2.1. Parasites

To ensure that all mussels were of a comparable age range, 15 mussels were selected according to a shell length between 30 to 50 mm. These represent specimens of similar physiology, also used in standardized bioassays (Ernst et al. 1991). Raw mussel were frozen and stored at -20 °C. After defrosting at room temperature (approx. 20-30 min) mussels were analyzed immediately. Length and width of each selected mussel was measured according to Seed (1968) to the nearest 0.1 mm using a vernier calliper. Mussels were opened, briefly drained on absorbent paper, and subsequently total wet weight was determined. Then, the soft body was removed and both shell and soft body were weighed (± 0.01 g) separately. The soft body was then placed on the bottom of a glass compressorium and the mantle, gills, food, adductor muscle and other tissues were dissected carefully and dispersed. The digestive gland was pulled apart and squeezed together with the other tissues using the cover glass of the compressorium. The preparations were examined under a stereo magnifying glass (10-50 magnification) with transmitting light for the presence of macro parasites. Parasite species were identified according to descriptions from the literature (e.g. Dethlefsen 1979; 1972, Lauckner 1983, Watermann et al. 1998) and infested organs listed. As freezing of the samples does not affect size of a trematod's metacercaria (Lepitzki et al. 1994), identification of trematodes was also reliable using frozen samples. In a final step all shells of the analysed mussels were inspected for the presence of shell-boring polychaets using the stereo magnifying glass.

40 mussels (30-50 mm) per sample site were analysed each sampling cycle to assess potential infestations with intracellular micro parasites, of the genus *Marteillia*. Fresh meat of 20 mussels was removed from the shell and glued separately on aluminium chucks before being frozen at –20 °C for kryostat-sectioning. To ensure a representative overview of potential infested organs, the frozen softbody was trimmed until digestive gland, gills, and palps appeared together in one tissue sections of the sample. Soft bodies of additional 20 mussels were removed and cut transversally according to international standard methods (Ifremer 2008) and subsequently used for smear preparations. Tissue sections and smear preparations were stained using *Haemacolor*® (Merck) before assessed by light microscopy.

2.2. Condition Index

30 mussels were used to calculate the condition index (CI) for all testing sites (data of 15 mussels used for macro parasite assessment added by 15 mussels used for histochemical analysis). For a direct comparison of CI and the parasite load only wet weights of tissues and shells could be used for the calculation (see below).

$$CI = \frac{Wet\ meat\ weight\ [g]}{Shell\ weight\ [g]} \text{ x } 100 \qquad (1)$$

2.3. Assessing lysosomal membrane stability (LMS)

Mussels between 3 and 5 cm in length were collected from the substrates and transferred immediately dry in cool boxes to the lab. The length and width of 15 mussels was measured to the nearest 0.1 mm using a vernier calliper. The mussels were opened; drained and total wet weight was determined (± 0.01 g). The soft body was then dissected, weighed (± 0.01 g) and put in cryo-vials each filled with 1 ml fish gelatine. Samples were controlled for remaining air bubbles and then frozen in liquid nitrogen. Subsequently, shells were weighed (± 0.01 g). Frozen soft body samples were fixed on pre-frozen aluminium chucks for subsequent cryostat-sectioning. Tissue sections of 10 µm were obtained using a cryotome (Microm, HM 500) with chamber temperature of -25 °C. Sections were stored for a maximum of 24 hours at -20 °C until processed for histochemistry.

The lysosomal membrane stability test was performed according to Moore et al. (2004). Serial cryostat sections were incubated at 37 °C in a 0.1 M citrate buffer, pH 4.5, containing 3 % NaCl to destabilize the membrane for increasing time intervals (2–50 min). After this acid labilisation, sections were incubated for 20 min at 37 °C in a medium containing the substrate Naphthol AS-BI N-acetyl β-D-glucosamide (Sigma) dissolved in 2-methoxy ethanol and low-viscosity polypeptide (Polypep, Sigma) dissolved in 0.1 M citrate buffer, pH 4.5 with 3 % NaCl. The lysosomal hydrolase N-acetyl ß-D hexosamidase catalyses the release of the Naphthol AS-BI group which undergoes a post-coupling reaction with the diazonium salt Fast Violet B (Sigma) dissolved in 0.1 M phosphate buffer (pH 7.4) leading to an insoluble bright violet reaction product. Following the colour reaction, samples were rinsed in running tap water, fixed in Baker's Formol, rinsed in distilled water and dried overnight in the dark at room temperature. Subsequently, slights were mounted in Kaiser's glycerine-gelatine.

2.4. Sectioning and staining for glycogen, lipofuscin and neutral lipid assessment

Tissue sections for the assessment of glycogen, lipofuscin and neutral lipids were obtained using the above described cryotome. To determine glycogen quantities duplicate sections were stained using the Perjod-Acid-Schiff (PAS) method (modified after Culling 1974). Therefore sections were fixated overnight in 10% formol before placed for two hours in an aldehyde blocking solution (2% sodium chlorite in 6% acetic acid). Then slides were washed in running tap water for 10 min, rinsed in distilled water for 2 min, placed in 1% periodic acid for 10 min and rinsed again in distilled water for 5 min. Then the sections were counterstained in Schiff's reagent for 8 min, bleached in sulfurous acid for 2 min and washed again in distilled water for 5 min. After few minutes drying slides were mounted in Euparal.

The accumulation of neutral lipids in the lysosomes was determined using the Oil-Red-O method modified after Lillie & Ashburn (1943). Duplicated sections were fixated in Baker`s Formol for 15 min, dipped three times in distilled water before washed in 60 % Triethylphosphate. Sections were then stained 15 min in Oil-Red-O solution (1% Oil Red O, 60% Triethylphosphate, pre-cooked for 5 min and filtered 1 time hot and 1 time cold). Stained sections were rinsed again for 30 sec in 60 % Triethylphosphate before washed with distilled water. Sections were dried shortly and mounted using Kaisers glycerine-gelatine.

The lipofuscin content of lysosomes was determined by using the Schmorl reaction modified after Pearse (1985). Duplicate cryostat sections (10µm) of the digestive gland were fixed 15 min in 4 % Baker's formalin then rinsed in distilled water before stained in a 1 % hydrous ferric chloride/potassium ferricyanide (1:1) solution. Tissues sections were stained for 20 sec, washed in 1 % acetic acid for 2 min, rinsed under tab water for 10 min and finally rinsed 3 times in distilled water. After a short drying period, sections were mounted with uv-resistant mounting medium (Histomount®). The optimal staining duration was pre assessed by a time series controlled with the light microscope to avoid staining of tissue background.

2.5 Microscopy

To assess the lysosomal membrane stability the maximum reaction product for N-acetyl β-D-Hexosamidase was determined by automatic measurement of number and percentage of dark stained lysosomes in the digestive tubules by the use of computer assisted image analysis (Zeiss, KS 300) combined with a light microscope (Zeiss, Axioskop) at 400 fold magnification. For contrast enhancement a green filter was applied. A long destabilization period indicates high lysosomal

membrane integrity and vice versa. Unclear results of labilisation series (no clear peaks, only 1 peak) were re-measured or excluded from further analysis.
Tissue sections stained with the Schmorl's and Perjod-Acid-Schiff method were quantitatively and objectively assessed for lipofuscin content using also computer assisted image analysis. The image analysis consisted of the above described microscope and camera and the software KS300 (Version 3.0, ZEISS). Staining intensity was measured using the same macro applied for LMS assessment. For both lipofuscin and glycogen assessment three black and white images were taken from each duplicate section of digestive gland tissue (6 measurements per individual) at 400 fold magnification. Similar to the LMS analysis a green filter for contrast enhancement was applied for PAS quantification. Neutral lipids were assessed semi quantitatively using the same microscope (Axioscope, ZEISS) with camera (MRc, ZEISS) coupled to a computer equipped with the software AxioVision (Version 4.6.3.0, ZEISS). Pictures were taken at 400 fold magnification and classified to 8 different categories of accumulation (0= no staining reaction, 7= maximum staining reaction).

2.6. Statistical analysis

Means and standard deviations (mean ± SD) of data of the macro parasite intensities and condition indices were calculated by using Microsoft Office Excel 2007. Box plots displaying LMS were calculated using Statistica 9.0 software. Differences of data on histopathology between sites and season were tested for significances with the software JMP (Version 7.0, SAS Institute Inc.). Data were analysed using a Kruskal-Wallis ANOVA on ranks with a Dunn's test as post-hoc test. The significance level was set at $p < 0.05$.

3. Results

3.1. Condition Index

According to the condition index values (CI) sites are divided into two groups (Fig. 2). Low CIs (CI 27.39 to 39-47) are found throughout the year with only moderate variances at the intertidal areas, whereas high indices (CI 61.21 to 113.79) are found at both culture sites. While the nearshore hanging culture JD showed an overall peak already in spring 2007 (CI 113.79) followed by a decrease of the CI down to 61.21 in autumn, the values of the offshore site stayed rather stable from spring to autumn with a minimum of change in winter time (CI 66.20). The mean values calculated for the whole sampling season showed the highest numbers for RS (94.5 ± 21.5 SD), followed by JD (82.97 ± 24.88 SD), NH (34.76 ± 5.56 SD), BS (32.58 ± 8.96 SD) and SY (31.38 ± 7.83 SD) (Fig. 2).

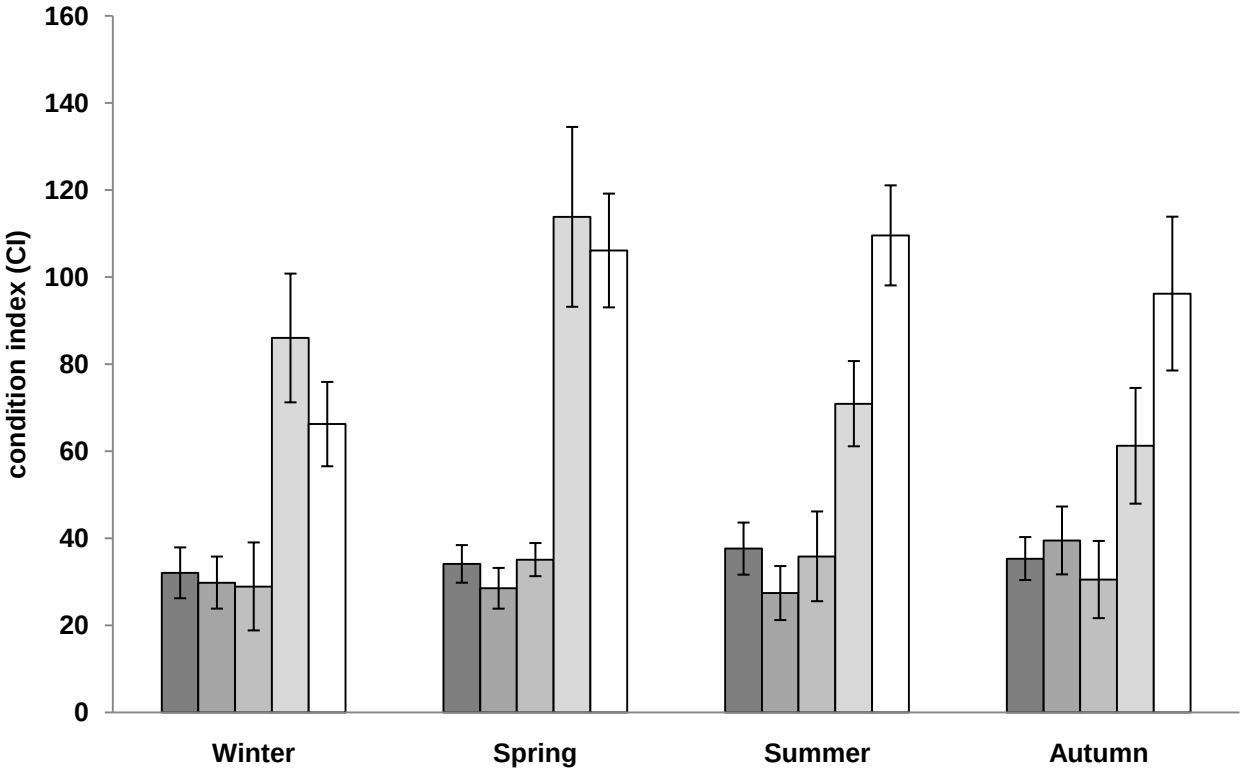

Fig. 2: Condition indices [CI] of blue mussels from five different sampling sites (NH [black], SY [dark grey], BS [grey], JD [light grey] and RS [white] over the season 2007 in the German Bight (n=30 per site and sample cycle).

3.2. Macro and micro parasites

Most macro parasites found in the tissues and organs of *M. edulis* belonged to four different native species (Krakau et al. 2006): *Mytilicola intestinalis* a copepod living as juvenile and adult individual in the digestive gland, two trematod species *Renicula roscovita* and *Himastla elongata* occurring as metacercarias in the gills, mouth palps and tubuli of the digestive gland or in the foot and other muscles, respectively. And last the Polychaet *Polidora ciliate* living in self drilled ducts of the shell of mussels. Other candidates such as *Modiolicula insgnis* and the species of the genus Gymnophallus occurred in less than 1 % of the cases and are not displayed. With the deployed sampling method (using a glass compressorium under a stereo magnifying glass) only adult *M. intestinalis* of >2.5 mm were found in the digestive gland.

The most common macro parasites showed a high prevalence of up to 100 % at the intertidal areas whereas the cultivated mussels are hardly infested (nearshore) or free of parasites (offshore) (Fig. 3). Prevalence of *M. intestinalis* from intertidal samples ranged from 45.0 % (NH), 68.33 % (BS) up to 86.67 % at SY (Fig. 3) with a mean intensity spreading from 0.87 ± 1.20 SD, 3.30 ± 2.30 SD and 3.22

± 2.76 SD individuals per mussel, respectively (Fig. 4b). At the nearshore cultivation area JD about 21.67 % of the mussels were infested by *M. Intestinalis* (Fig. 3) with an average of 0.33 ± 0.73 SD individuals (Fig. 4b).

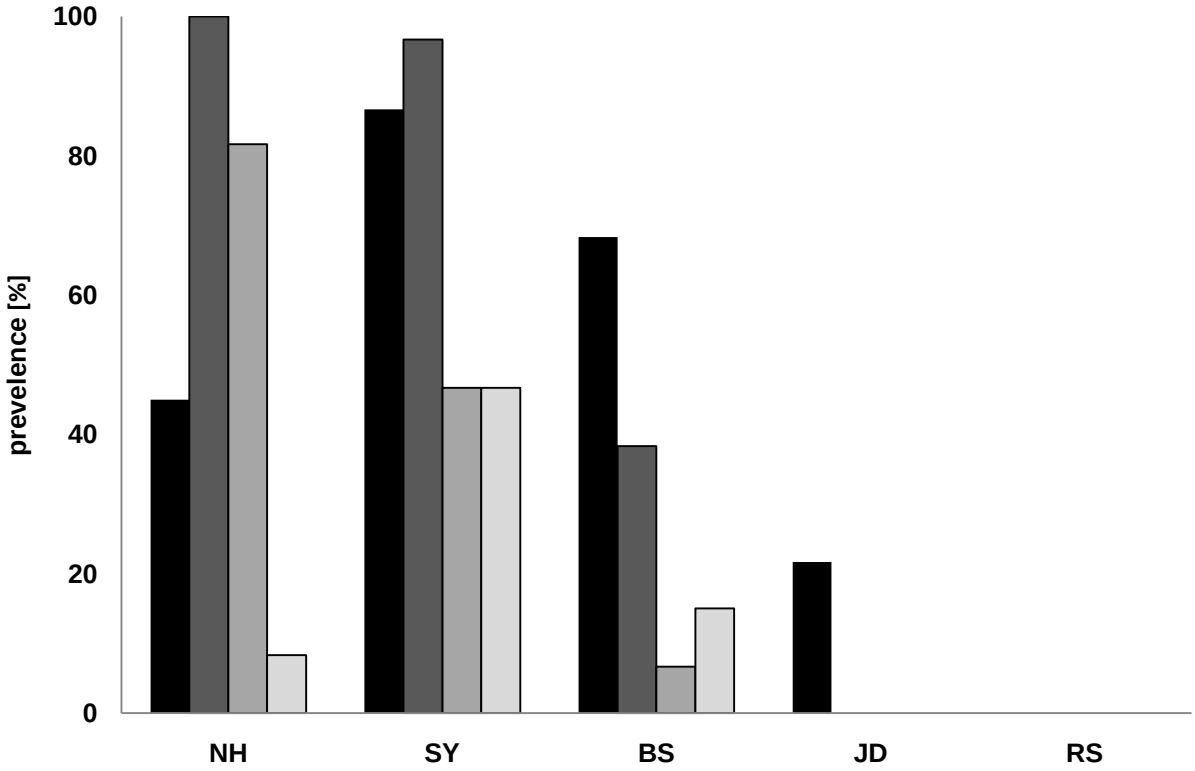

Fig. 3: Prevalence [%] of macro parasites *M. intestinalis* (black), *R. roscovita* (dark grey), *H. elongate* (grey) and *P. ciliata* (light grey) found in blue mussel according to five sampling site (n=60 per site) in the German Bight during the season 2007.

Trematods occurred in two species in intertidal areas. There, *R. roscovita* exhibited a prevalence up to 96.67 % at SY and 100 % at NH (Fig. 3) together with high mean intensities of 90.52 ± 91.05 SD and 197.28 ± 331.40 SD individuals per mussel, respectively (Fig. 4c). At the SY sampling site mass infestations with >1000 *R. roscovita* were also observed. BS showed low intensities of an average of 5 ± 13.80 SD metacercarias of *R. roscovita* per mussel in about 38.33 % of the samples (Fig. 3 & 4c). *Himastla elongata* the second trematod specie found as metacercarias occurred, similarly to *R. roscovita,* only at intertidal sites. In this case prevalences were highest in NH (81.67 %), followed by SY (46.67 %) and BS with 6.67 % of infested mussels (Fig. 3). Intensities were low and ranged from 8.28 ± 9.22 (NH), to 2.67 ± 5.34 SD (SY) and 0.22 ± 1.04 SD at BS (Fig. 3 & 4c).
Similarly to the three other parasite species, *P. ciliata* occurred only at intertidal sites. Prevalence was high in SY (46.67 %), moderate at BS (15.00 %) and low at NH (8.33 %) (Fig. 3). Intensities were also low and ranged between 0.10 ± 0.35 SD at NH, 2.02 ± 4.00 SD at SY and 0.20 ± 0.55 SD at the sample site of BS (Fig. 4a).

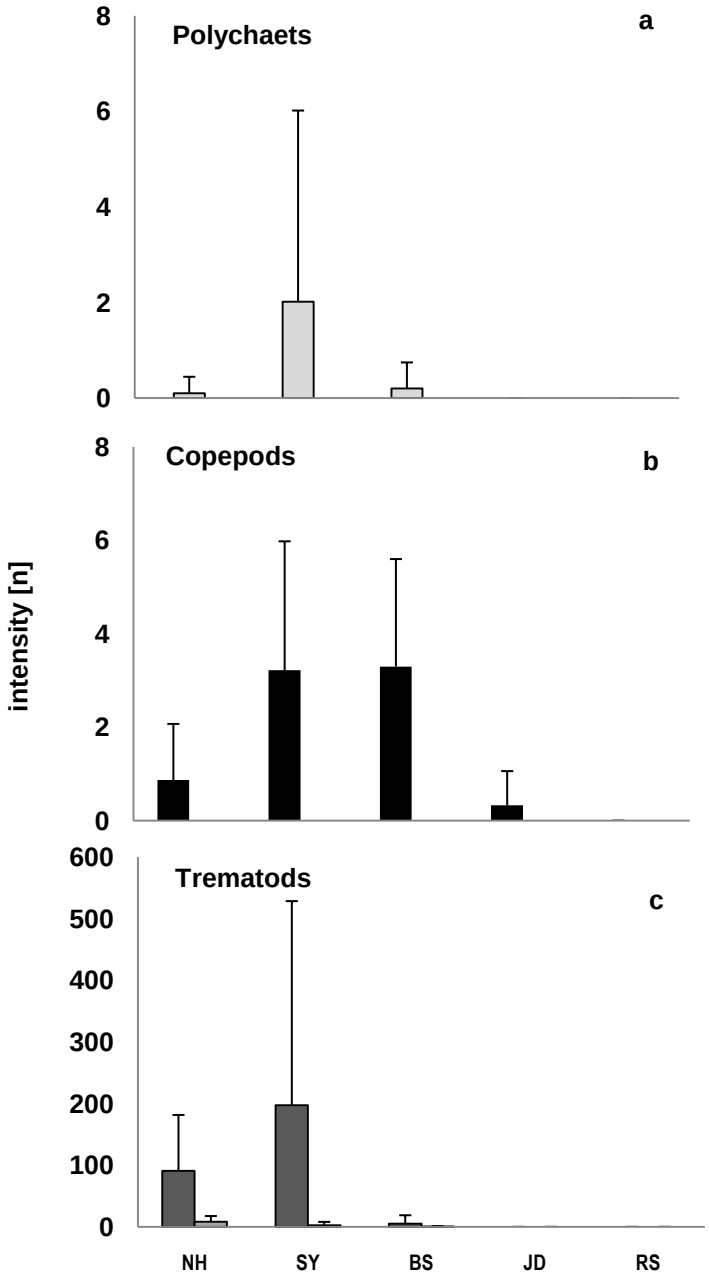

Fig. 4a-c: Intensity [n] of macro parasites (a shell boring Polychaet/ *P.ciliata* [light grey], b Copepod/ *M. intestinalis* [black], c Trematods/ *R. roscovita* [dark grey]; *H. elongate* [light grey]) in blue mussels of five sampling sites (n=60 per site) in the German Bight in the year 2007.

Adult *M. intestinalis* inhabit only the hind gut of the digestive gland, whereas *R. roscovita* occurred in the tubuli of the digestive gland (59 %) and in the gills or pulps (35 %) of the mussel. The second trematod species *H. elongata* is found mainly in the foot (78 %) and in other muscular tissues (15 %) (Tab. 1). The most invested organ by macro parasites was the digestive gland, where *M. intestinalis* and *R. roscovita* were found, mouth palps and gills were infested by *R. roscovita* and the foot was infested by mainly *H. elongata* and to a certain extent also *R. roscovita* (Tab. 1).
All organs and tissues of the investigated samples from all five different sample sites were free of *M. refrigens* throughout the year 2007.

Tab.1: Infestation [%] of mussel (n=300) organs by most common parasites of blue mussels from five sampling sites of the German Bight (2007).

	Digestive gland	Gills/Palps	Foot	Muscle	Shell
M. intestinalis	100	-	-	-	-
R. roscovita	59	35	3	3	-
H. elongata	6	1	78	15	-
P. ciliata	-	-	-	-	100

3.3. Lysosomal membrane stability

The results of the LMS-analysis for the five sampling locations according to site are shown in Figure 5a-j. Intertidal sites (5 a/b, c/d, or e/f) did not show any significant differences over the sampling season for peak 1 and 2. At both sites for hanging cultivated mussels (5 g/h and 5 i/j), however, peak 1 values for spring and summer differed significantly. Values for peak 2 did not show any significant differences. Both cultivation sites display comparable trends or peak 1 and 2 showing the lowest labilisation values in spring followed by an increase in summer. Autumn and winter sample at both sites stayed mostly stable on intermediate levels. The two intertidal sites NH and SY show contrary trends with higher values for peak 1 and 2 in spring followed by a decline of values in summer for both peaks. BS is comparable with the both cultivation sides, however, only concerning values for peak 1.

In Figure 6a-h labilisation values for the 5 different sites were sorted according to season. The only significant difference was detected in summer (Fig. 6e) between peak 1 of NH and JD.

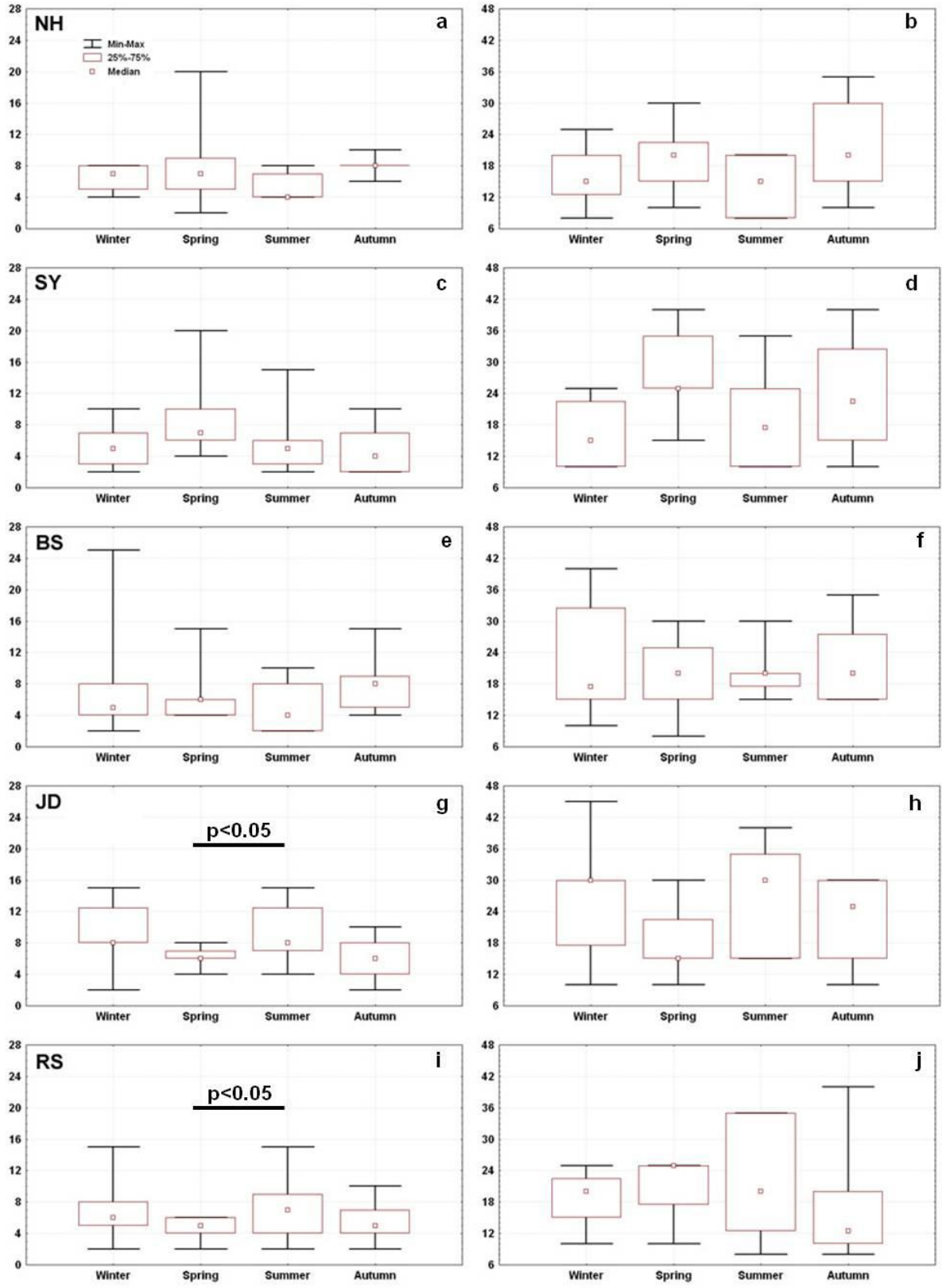

Fig 5a-j: Box-Whisker plots of NH peak 1 (a) and 2 (b), SY peak 1 (c) and 2 (d), BS peak 1 (e) and 2 (f), JD peak 1 (g) and 2 (h), and RS peak 1 (i) and 2 (j) of four consecutive sampling cycles of the season 2007 from the German Bight. Differences for peak 1 between spring and summer at JD (g) and RS (i) are significant ($p < 0.05$).

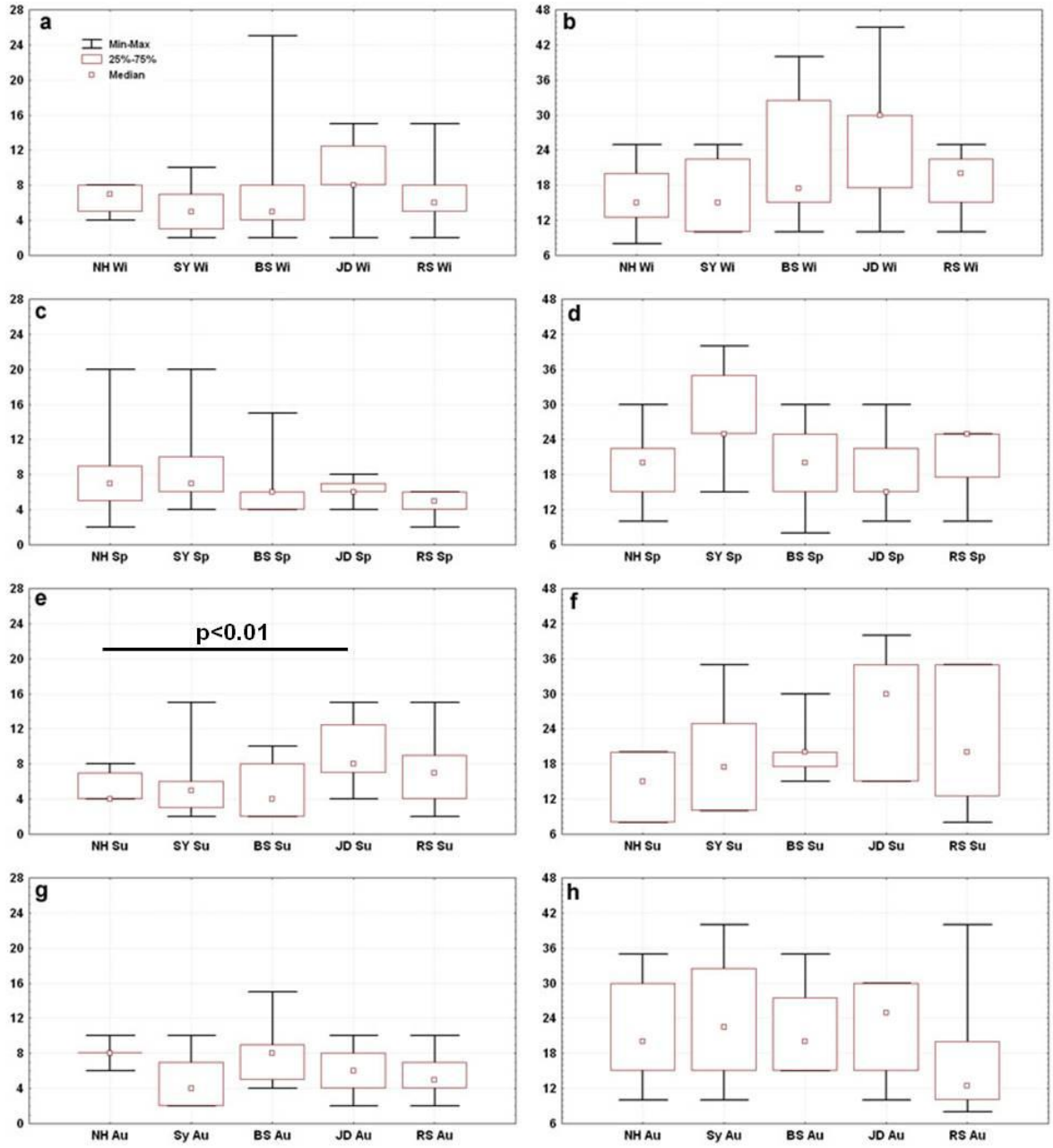

Fig 6a-h: Box-Whisker plots of winter peak 1 (a) and 2 (b), spring peak 1 (c) and 2 (d), summer peak 1 (e) and 2 (f) and autumn peak 1 (i) and 2 (j) from five different sites (NH, SY, BS JD and RS) of four consecutive sampling cycles of the season 2007 from the German Bight. Differences for peak 1 between NH and JD in summer (e) are significant ($p < 0.05$).

3.4. Glycogen quantification

The results of the quantification of glycogen in lysosomes of the digestive gland are displayed in Figure 7a-d. Throughout the year mussels from all sampling sites show high glycogen concentrations, however, - with only one exception of SY in autumn (7d) - always highest for mussels from the submerged cultivation sites. At the cultivation sites values were higher for RS in winter and autumn whereas JD showed highest values in spring and summer. More variable were the values from the 3 intertidal sites. Significant differences were detected between SY vs. JD, SY vs. RS and SY vs. NH in summer (7b) and between SY vs. NH and SY vs. BS in autumn (7d).

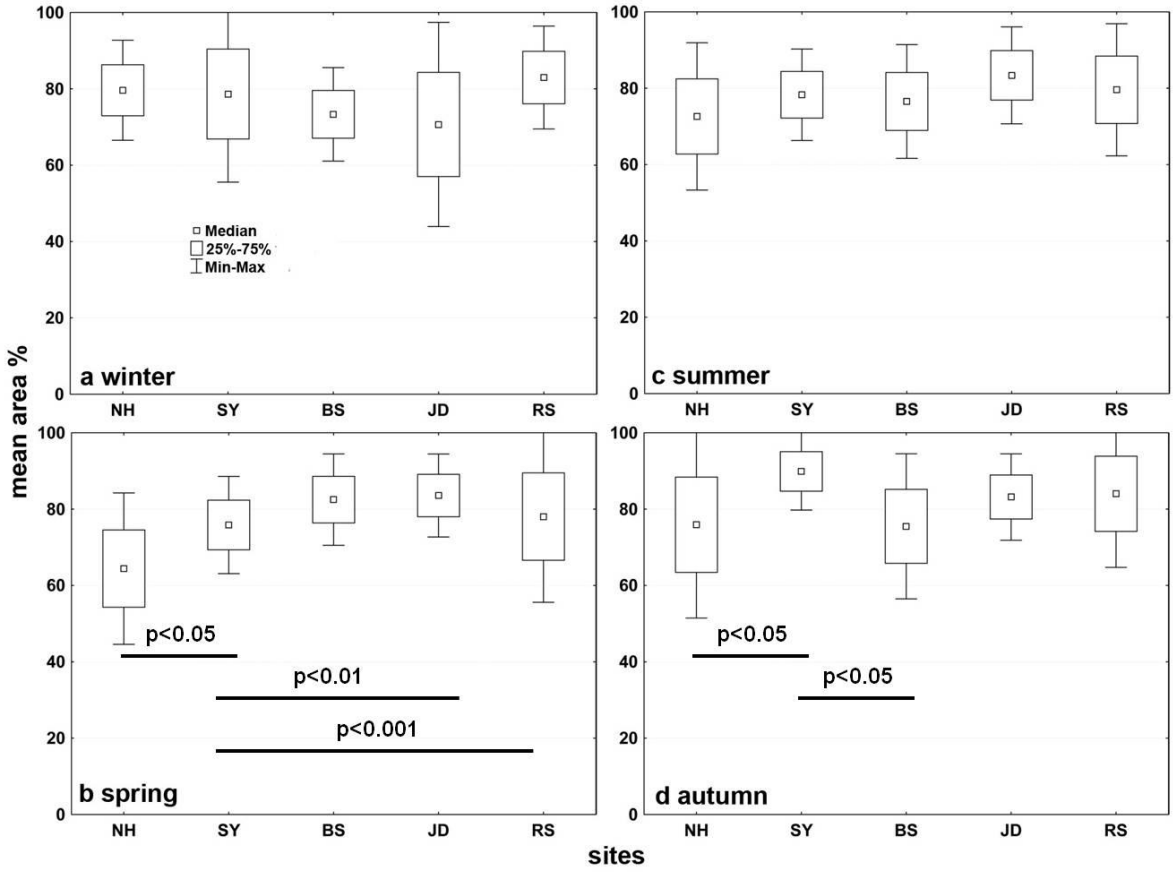

Fig 7a-d: Box-Whisker plots for lysosomal glycogen concentrations in mean area % of winter (a), spring (b), summer (c) and autumn (d) from five different sites (NH, SY, BS JD and RS) of the season 2007 from the German Bight. Differences between SY vs. JD, SY vs. RS and SY vs. NH in summer (7b) and between SY vs. NH and SY vs. BS in autumn (7d) are significant ($p < 0.05$).

3.5. Lipofuscin assessment

The results for the concentrations of lysosomal lipofuscin from tissue of the digestive gland of blue mussels are displayed in Figure 8a-d. Lowest values with biggest variances especially for intertidal mussels were detected in winter. Values for all sampling sites increase in spring and summer and show a slight decrease in autumn. Values from the submerged cultivation sites are already high in winter and vary throughout the year only slightly on a high level. Especially the offshore area RS showed extremely high values with low variances. Significant differences were detected in winter between RS vs. SY, RS vs. NH and BS vs. SY. In spring values differed significantly between RS vs. JD, RS vs. SY and BS vs. NH. The analysis in summer resulted in significant differences for RS vs. JD, RS vs. BS, RS vs. NH and BS vs. SY. In autumn significant differences were detected between NH vs. SY, NH vs. BS and NH vs. JD.

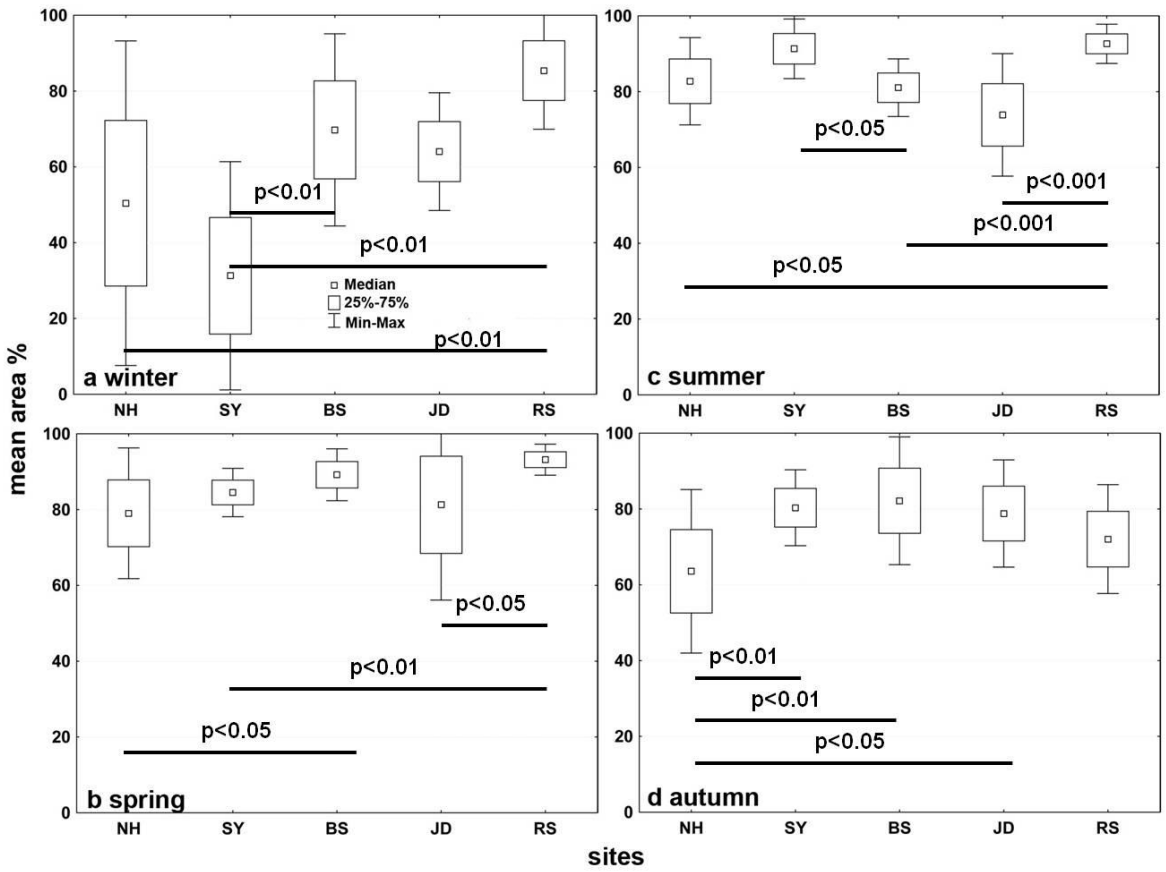

Fig 8a-d: Box-Whisker plots for lysosomal lipofuscin concentrations in mean area % of winter (a), spring (b), summer (c) and autumn (d) from five different sites (NH, SY, BS JD and RS) of the season 2007 from the German Bight. Significant differences were detected in winter (a) between RS vs. SY, RS vs. NH and BS vs. SY. In spring (b) values differed significantly between RS vs. JD, RS vs. SY and BS vs. NH. The analysis in summer (c) resulted in significant differences for RS vs. JD, RS vs. BS, RS vs. NH and BS vs. SY. In autumn (d) significant differences were detected between NH vs. SY, NH vs. BS and NH vs. JD. Significance level for all statistical analyses was p< 0.05.

3.6. Neutral lipids assessment

The results of the semi quantitative assessment of the lysosomal accumulations of neutral lipids are displayed in Figure 9 a-d. There is a strong and static increase in the accumulation values at all sites over the sampling period form winter to autumn. Similar to the glycogen and lipofuscin assessment values were highest at the submerged cultivation areas with an all year maximum at RS. Differences between sites were significant at RS vs. JD and RS vs. BS in winter (9a). In spring (9b) significantly different values were detected between RS vs. BS, RS vs. SY and JD vs. BS. Summer (9c) values differed significantly between RS vs. BS, RS vs. SY, JD vs. BS and JD vs. SY. In autumn (9d) both cultivation sites differed significantly from all intertidal sites.

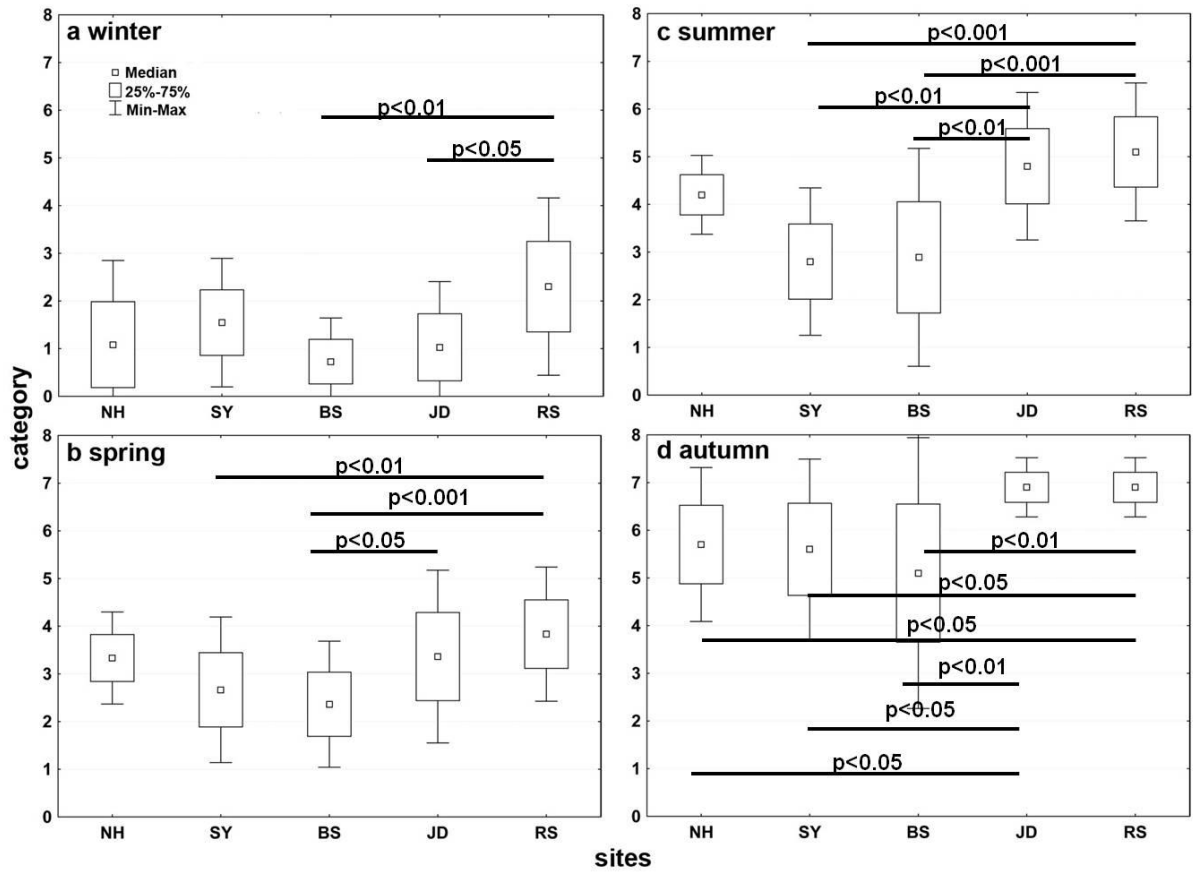

Fig 9a-d: Box-Whisker plots for lysosomal neutral lipid concentrations in categories (0= no neutral lipids and 7=maximum concentration of neutral lipids) of winter (a), spring (b), summer (c) and autumn (d) from five different sites (NH, SY, BS JD and RS) of the season 2007 from the German Bight. Significant differences were detected in winter (a) between RS vs. JD and RS vs. BS. In spring (b) values differed significantly between RS vs. SY, RS vs. BS and JD vs. BS. The analysis in summer (c) resulted in significant differences for RS vs. BS, RS vs. SY, JD vs. BS and JD vs. SY. In autumn (d) significant differences were detected between RS vs. BS, RS vs. SY, RS vs. NH, JD vs. BS, JD vs. SY and JD vs. NH. Significance level for all statistical analyses was p< 0.05.

In Figure 10.1a-d-10.5a-d photos used for the semi-quantitative assessment are displayed showing the seasonal variances at the different sites and their significance levels. At all sites there is a significant increase of neutral lipid accumulation between winter and autumn (Fig. 10.1a/d, 10.2a/d, 10.3a/d, 10.4a/d and 10.5 a/d). With the exception of SY (Fig. 10.3a/c) values are also significantly different between winter and summer Fig. 10.1a/c, 10.2a/c, 10.4a/c and 10.5a/c).

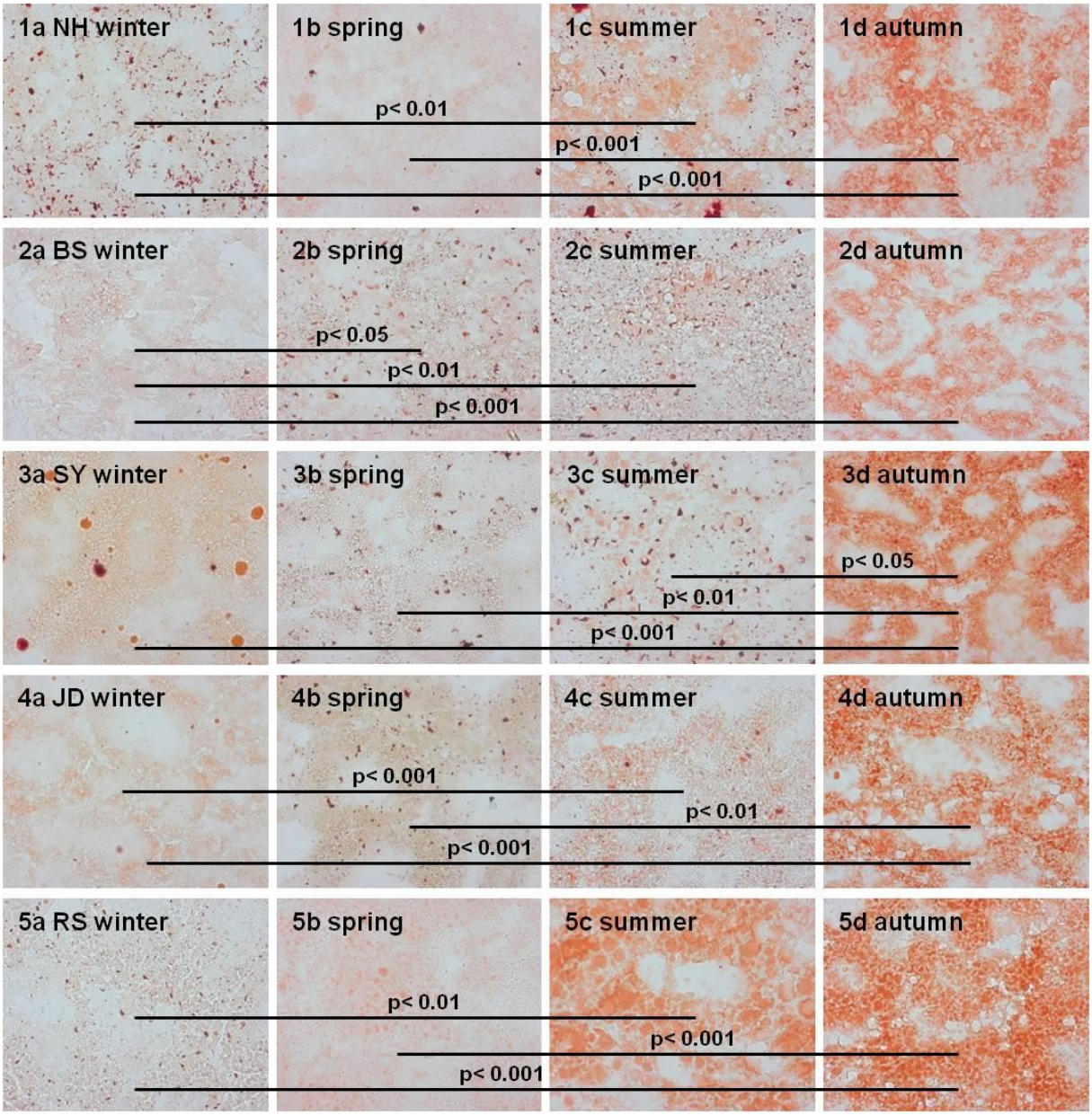

Fig 10.1a-d-10.5a-d: Box-Whisker plots for lysosomal neutral lipid concentrations in categories (0= no neutral lipids and 7=maximum concentration of neutral lipids) of winter (a), spring (b), summer (c) and autumn (d) from five different sites (NH, SY, BS JD and RS) of the season 2007 from the German Bight. Significant differences were detected in winter (a) between RS vs. JD and RS vs. BS. In spring (b) values differed significantly between RS vs. SY, RS vs. BS and JD vs. BS. The analysis in summer (c) resulted in significant differences for RS vs. BS, RS vs. SY, JD vs. BS and JD vs. SY. In autumn (d) significant differences were detected between RS vs. BS, RS vs. SY, RS vs. NH, JD vs. BS, JD vs. SY and JD vs. NH. Significance level for all statistical analyses was p< 0.05.

4. Discussion

It is widely believed that cultivation of organisms under offshore condition will lead to increased growth and better product quality since e.g. a better water quality, less temperature variability, better O_2-conditions and lower microbial or contaminant loads can be expected due to the distance to shore and the resulting dilution effects. The definition of the term offshore focuses on hydrographical or socio economic prerequisites: (1) the full exposure to all environmental conditions (Ryan 2005) and (2) a minimum distance of eight nautical miles to shore (Buck 2007) and reduced stakeholder conflicts (Dahle et al. 1991). The data of this study show, however, that a differentiated perspective is necessary in marine areas such as the German Bight. The test area RS fulfils the offshore definition for exposure and distance to shore. Regarding macro parasites, growth and aesthetical appearance, mussels of high quality can be produced offshore. Except for macro parasites the same is true for nearshore hanging cultivated mussels. However, the biodiagnostic approach to determine health by testing the lysosomal membrane stability and the concentration of glycogen did not detect differences for the 10 months duration time of the experiment between intertidal and hanging cultivated mussels near- and offshore. Other biomarkers such as the lysosomal lipofuscin concentration and the accumulation of lysosomal neutral lipids give even evidence that hanging cultivated mussels are exposed to more environmental stress than intertidal mussels.

Data of the Federal Hydrographic Agency (BSH, Hamburg) show that pollutants and heavy metals were found in the water column and suspended matter of the whole German Bight. Highest concentrations of the five different sampling sites were detected offshore at RS especially in autumn 2007. An explanation for these findings might be that the offshore site RS is located at the south-west edge of an intermixing zone of estuarine run-offs of the rivers Weser and Elbe (BSH 2009). Due to the more extensive drainage area of the Elbe the river is burdened with higher loads of contaminants (BSH 2005, UBA 2009), leading to the paradox situation that pollutant concentrations are higher offshore than nearshore in this region. This effect is more pronounced in autumn, since run-off is increased by higher precipitation. According to OSPAR (2008) the southern part of the North Sea including the German Bight remains in an unacceptable status, showing concentration levels of heavy metals and organic pollutants in the sediment and in biota with assumed risks to the environment at population, and community level. Although some pollutants (e.g. PCB) and heavy metals (e.g. lead and mercury) show trends of decrease in concentrations, the Southern North Sea including the German Bight is still one of the most polluted marine areas of the OSPAR regions (OSPAR 2008).

Infestations with parasites in the German Bight, as determined in this study, followed the pattern described in other publications (e.g. Buck et al. 2005, Brenner et al. 2009). Macro parasites were absent in offshore mussels but present in -over the sampling season- increasing prevalence and intensities in nearshore samples. The offshore situation can be explained by: (i) the absence of intermediate hosts (e.g. *Littorina* spp. for trematod species), which are restricted to nearshore water habitats, (ii) the distance from the host populations, resulting in dilution effects and (iii) the poor swimming capacities of parasites' planktonic reproduction stages (e.g. *M. intestinalis*) (Davey and Gee 1988). According to the findings of Brenner et al. (2009) intertidal individuals from wild mussel beds showed high infestations rates regarding number of parasites and number of parasitic species. The mussels from the nearshore area JD cultivated off-bottom in the vicinity of these highly affected populations were infested moderately from the second year on, however only by the copepod *M. intestinalis*. Interestingly, the displayed significant differences between parasite infestations rates of intertidal and hanging cultivated mussels are -similar to the difference of the condition indices which follow the same pattern- not detectable in the LMS values. This is particularly astonishing, since infestations with parasites are concentrated on the digestive gland. Mass infestations with parasites are affecting functionality and health of this important organ and this stress should be measurable by applying the test on lysosomal membrane stability.

The fact that all investigated mussels regardless of their habitat conditions, sampling season or year from all over the German Bight exhibit similar low LMS values (5-8 min) provides strong evidence that the cause for the indicated stress is the same for all sampling sites. The described relatively homogenous water body burdened with contaminants is the most conceivable reason for these findings. According to Viarengo et al. (2007) these animals are considered as severely stressed,

already exhibiting pathologies. If this is true, the coastal water up to 12 nautical miles distance to shore in the German Bight poses a severe risk to the health of blue mussels. The only significant differences in LMS detected were at both cultivation sites between spring and summer, most probably due to reproductive processes, known to reduce fitness and health of the spawning animals.
Other biomarker sensitive for environmental stress like the assessment of lipofuscin and neutral lipids content showed, however, seasonal variations and were sensitive for the habitat conditions on the sampling sites. The semi quantitative assessment of neutral lipid displayed a pronounced increase of lipid concentrations from winter to autumn most prominently at the cultivation sites. The lipofuscin concentrations achieved their highest levels after a short increase already in spring and remained for the rest of the year on high level. Similar to the neutral lipid assessment highest lipofuscin values were detected at the cultivation sites, especially at RS. Mussels living suspended hanging in the water column can feed permanently. The good food supply at hanging cultivation sites explains the rapid growth and high condition indices, however, also seem to lead to an increased accumulation of pollutants incorporated together with the food. Intertidal mussels in the German Bight interrupt feeding for about twelve hours a day during time of emerging. In this phase an effective excretion of metabolites seem to take place.

Outlook

The results of this study elucidate the potentials and some constraints of the deployed biomarker. Like described in many other studies the LMS is sensitive for anthropogenic pollution in the marine environment, however, not a suitable tool to measure the health effects of enhanced parasite infestation rates. Other biomarker like neutral lipids and lipofuscin concentration display a strong seasonality and show remarkable differences according to habitat conditions. The enhanced metabolism of hanging cultivated mussels lead to a significant stronger accumulation of metabolites. These differences between intertidal and submerged mussels should be considered if monitoring data about environmental stress have to be interpreted. The same is true, if e.g. intertidal mussels are used for caging experiments in subtidal testing areas. This habitat change may cause changes in the metabolism and lead to an increased accumulation although water quality and pollution stress remained the same.

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